IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: KITAMURA=1

In re application of:	)	Art Unit: 1651
Hidetomo KITAMURA	)	Examiner:
Appln. No. 09/380,372	)	Washington, D.C.
Filed: September 1, 1999	)	
For: NOVEL CELL LINES AND	)	
SCREENING METHODS...	)	

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Hidetomo KITAMURA, a Japanese citizen, residing at 135, Komakado, 1-chome, Gotenba-shi, Shizuoka, 412-8513, Japan, hereby declare that I am the inventor of the above-entitled patent application, and that I received a D.V.M degree from Hokkaido University Faculty of Veterinary Medicine in March 1991.

I declare also that I have been employed by Chugai Seiyaku Kabushiki Kaisha, the assignee of this application,

and have been engaged in pharmaceutical research since April 1991 and that I work as a researcher for Pharmaceutical Research Laboratory II of Chugai Seiyaku Kabushiki Kaisha.

I also declare that I have read all of the Official Actions pertaining to the above-entitled application, and am familiar with each of the references cited in the Official Actions by the Examiner.

I declare further that the following experiment was conducted under my supervision and that the result is true and correct to the best of my knowledge.

Experiment

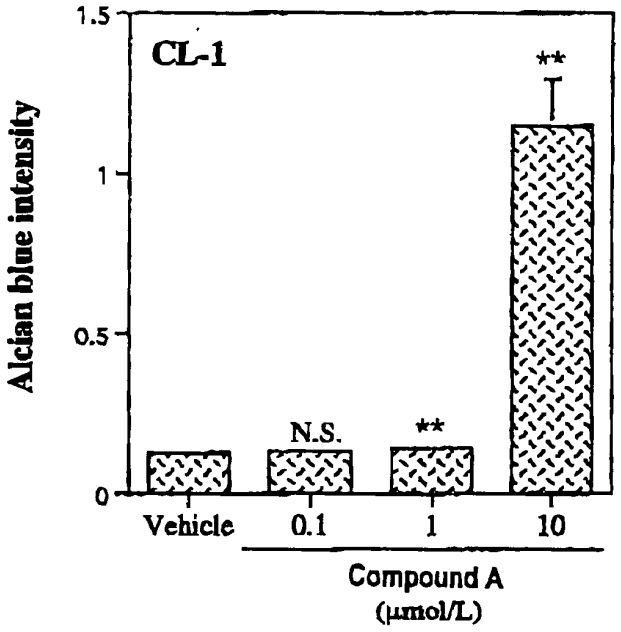
1. Methods

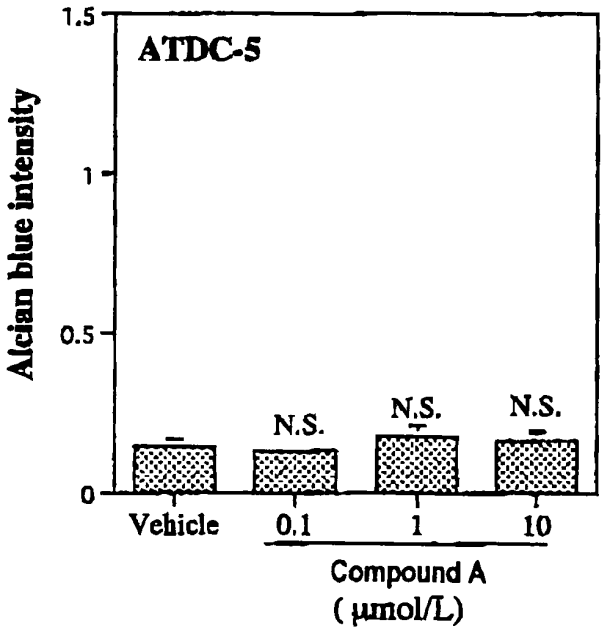
The CL-1 cells of the instant invention and the ATDC5 cells were grown to confluent on respective 24-well culture plates (5,000 cells/well, CORNING) in α-MEM (GIBCO) containing 10% fetal calf serum (INTERGEN), L-ascorbic acid (50 μg/mL, Wako pure chemicals), penicillin (100 U/mL, Meiji Seika Kaisha) and streptomycin (100 μg/mL, Banyu), and then treated for 7 days with either of vehicle control (1% ethanol) or Compound A (at 0.1, 1.0 or 10 μmol/L). The ATDC5 cell line was obtained from embryonic carcinoma and has been widely used in the art as a chondrocyte precusor in studying chondrocyte differentiation. Compound A is a chondrogenic compound

disclosed in page 17 of EP1156037 A1. The cell layers of the CL-1 cells and ATDC5 cells were fixed with 4% paraformaldehyde (Wako pure chemicals) at 4°C overnight. After washing with distilled water, the fixed cell layers were treated with HCl (0.1 mol/L, Wako pure chemicals) for 3 minutes and stained with an alcian blue solution (pH 1.0, EM Science) overnight at room temperature. After washing with HCl (0.1 mol/L), alcian blue pigment was extracted from each of the fixed cell layers by guanidine HCl (6 mol/L, 300 µL/well, Wako pure chemicals) overnight at room temperature. For evaluating the alcian blue intensity, absorbance of the extracts at 620 nm was measured by a microplate reader (MULTISKAN).

2. Results

Effect of Compound A on CL-1 and ATDC-5





**: p<0.01 vs Vehicle (1% ethanol)
N.S.: Not Significant
Student t-test

As is apparent from the above graphs, the alcian blue intensity significantly increased in the CL-1 cells treated with Compound A at 10 μmol/L, indicating that cartilage formation of the CL-1 cells was significantly promoted by the treatment of Compound A. On the other hand, the treatment of ATDC5 cells with Compound A did not increase the alcian blue intensity.

Therefore, it is concluded that Compound A promoted *in vitro* cartilage formation of the CL-1 cells of the present invention, which cells were derived from an adult animal. Compound A, however, did not promote *in vitro* cartilage formation of the ATDC5 cells, which were isolated from a differentiating culture of an mouse embryonal carcinoma cell line AT805 (Atsumi et al., Cell Diff. Develop., 30: 109-116, 1990, and Takeichi et al., Develop. Biol., 87: 340-350, 1981).

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false

statements may jeopardize the validity of the application or any patents issued thereon.

Dated this 27 th day of February 2003

Hidetomo KITAMURA